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PROGRESS REPORT

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ULTRASENSITIVE DETECTION OF CHEMICAL SUBSTANCES

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PRINCIPAL INVESTIGATOR
ROGER DEAN, Ph.D.
UNIVERSITY OF NEW MEXICO
SCHOOL OF MEDICINE
ALBUQUERQUE, NEW MEXICO 87131

(505) 277-5987

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Our proposal "Ultrasensitive Detection of Chemical Substances" submitted to the Defense Advanced Research Projects Agency in September 1983, proposed the development of an ultrasensitive detection system for chemical agents of small molecular size. This system would have an increase in sensitivity over current immunological detection systems and would allow acquisition of information which cannot be obtained with current detection sensitivities.

Our approach to ultrasensitive detection is characterized by trapping a molecule of interest (ligand) with a trapping molecule and then specifically identifying the complex with an antibody that carries an easily measured signal. In principal this method shares many common points with so-called "sandwich" assays where the ligand is trapped initially by a first antibody and identified with a second antibody. There is, however, one important difference. Our trapping molecule is not an antibody, but rather an enzyme, AChE, which is the target for organophosphate toxins. This enzyme is inactivated by organophosphate toxins by covalent irreversible binding of the toxin. Thus AChE traps the molecules (organophosphates) that we intend to detect. This method of trapping and localizing molecules has several advantages over typical sandwich assays, one of the most important being the ability to detect chemical species of small size.

In our proposal we set out several specific tasks for accomplishment during the period of our contract. These goals when condensed can be stated as three basic requirements for the success of an ultrasensitive assay of the type we describe. These requirements are:

1. To produce monoclonal antibodies specific for the AChE-DFP complex as opposed to antibodies against AChE alone.

2. To eliminate non-specific signal generation (noise) in our ultrasensitive detection test.

3. To enhance specific binding through increased antigen/antibody accessability and, most importantly, through stabilization of the antibody/antigen bond.

In our first goal of producing and selecting monoclonal antibodies with the appropriate specificity, we have been most successful. In the first year of our contract and extension we were able to select clones from five hybridomas with specificity for the AChE-DFP complex. This was reported in our final report of that period. The importance of this success is that it demonstrates the feasability of producing an antibody with the required specificity which our entire concept is dependent on. During our most recent contract year, we have worked to characterize these monoclonal antibodies and to produce more

hybridomas with the required specificity. Characterization includes antibody subclass determination by immunoelectrophoresis and determination of the intrinsic affinity constants by ELISA methods.

Recently we measured the intrinisic affinity constant of protein A purified antibody specific for AChE-DFP. The calculated affinity constant was 1.12×10^{8}, $mole^{-1}$, which is a reasonably high affinity. Since affinity constants reflect the dissociation and association constants and association constants will be similar for antibodies to a particular antigen, we can test our monoclonal antibodies for their relative strength of binding using affinity constant calculations. As monoclonal antibodies continue to be produced, they will be selected for high affinity constants. This effort will require significant numbers of monoclonal antibodies and is important to our third requirement of stabilizing the antigen/antibody bond.

Our second major requirement is the reduction of noise in our ultrasensitive detection test. This aspect of our contract has received the bulk of our attention during the past contract year. In our early tests we found and reported noise levels of fluorescent beads bound non-specifically to polystyrene solid supports between 600 and 10^{4} beads/cm^2. In more recent studies we found an average of 200 beads/cm^2 bound non-specifically in our test system utilizing rabbit IgG as an antigen and goat-anti-rabbit antibody as the reacting antibody bound to the fluorescent beads. This level of residual binding was very resistant to removal by washing, even with the most stringent reagents. Our experiments also indicated the non-specific binding was not due to interactions between proteins or between proteins and the polystyrene support. Apparently the interaction is between the polystyrene bead and the polystyrene of the plate surface. Recently we studied the interaction between beads whose surface carries one of three types of functional groups (amine, carboxyl, or succinamide) and polystyrene plates treated so as to present surfaces with particular functional groups. These results are presented in Table 1. It can be seen that the greatest difference occurs when beads are presented to the polystyrene surface in a non-ionic solution. Variations are observed in other combinations of beads and plates, but nothing as dramatic as the incubations in deionized water. We have also tested surfaces other than polystyrene. In the case of gel-bond surfaces rather than polystyrene, indications are that noise levels can be reduced to less than 10 beads/cm^2 without great difficulty.

To put these numbers in perspective, a hypothetical detection device with a surface area of 1 cm^2, as described in our proposal, would have less than 40 units of noise using polystyrene as a support and less than 10 units of noise in the case of a gel-bond support. These levels of noise are sufficiently low to allow further development of the ultrasensitive assay.

More importantly, we have gained considerable understanding of the causes of noise in our system. Understanding of the source of noise is important because noise levels are dependent on the concentration of reactant (beads) and the time of reaction as in any other chemical process. Thus noise levels will change as variables are manipulated to insure maximum specific binding.

Work on optimizing stabilizing specific bonds has been limited due to our effort to lower the noise levels. However, we have begun to work in two important areas. First, we have completed our first study with monoclonals against AChE-DFP. This study was done before noise levels were reduced and noise problems were encountered. However, the study indicated specific binding with two of the monoclonals tested. Second, studies have begun with the gel-bond solid support to bind antigen to it and determine levels of specific interaction. At this time we find no specific binding between the antigen surface and the antibody coated bead. This may be due to the antigen being buried in the agar matrix of the gel-bond surface. In the coming months we will focus on specific binding.

In addition to the major requirements and objectives discussed above, a number of accomplishments of a lesser, but important, value to the project have been completed. Listed below are some of the developments that we believe have and will continue to make important contributions to this contract.

1. The rabbit IgG/anti-rabbit test system has been standardized for both noise levels and specific binding. This serves as a reference for further testing.

2. Reverse centrifugation has been developed and used successfully. This procedure can be used to quantitatively measure the strength of both specific and non-specific binding of beads to plates.

3. We have measured the amount of protein to plates at saturation of input protein with polystyrene plates given various surface treatments. Protein binding to gel-bond film has also been measured. Similarly, using radio-labelled protein we have quantitated the amount of protein binding to MX covasphere beads.

4. The first device for testing sheet plastics other than polystyrene was made.

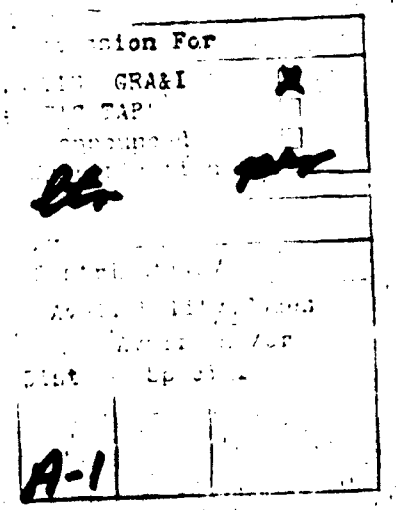

TABLE 1

BEADS	PLATE TREATMENT				
	Untreated	Sulfonated Surface	Animated Surface	Poly-L-lysine Coated	Untreated Surface Non-buffered H_2O
CX (carboxyl surface)	4.5×10^3	4.8×10^3	5.1×10^2	1.4×10^3	35.38
FX (Amine Surface)	6.9×10^3	2.9×10^4	2.1×10^4	6.2×10^2	-
MX (N hydroxysuccinamide)	6.3×10^3	7.7×10^2	1.5×10^3	8.2×10^3	-

All figures are beads/cm^2

All incubations of beads are carried out in PBS buffer, except as where indicated.